Plasma citrulline concentration: a surrogate end point for radiation-induced mucosal atrophy of the small bowel. A feasibility study in 23 patients.

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CLINICAL INVESTIGATION **Normal Tissues**

PLASMA CITRULLINE CONCENTRATION: A SURROGATE END POINT FOR RADIATION-INDUCED MUCOSAL ATROPHY OF THE SMALL BOWEL. A FEASIBILITY STUDY IN 23 PATIENTS

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Purpose: Plasma citrulline, a nitrogen end product of glutamine metabolism in small-bowel enterocytes, was suggested as a marker of radiation-induced small-bowel epithelial cell loss in mice after single-dose whole-body irradiation. Our objective was to evaluate the feasibility of citrulline as a marker for radiation-induced small-intestinal mucosal atrophy in patients during and after abdominal fractionated radiotherapy.

Methods and Materials: Twenty-three patients were studied weekly during treatment and at intervals of 2 weeks and 3 and 6 months after treatment by postabsorptive plasma citrulline concentration and clinical toxicity grading. The interrelationship between these variables and the correlation with small-bowel dose and volume parameters were investigated.

Results: During fractionated radiotherapy, citrulline concentration significantly decreased as a function of the radiation dose ($p < 0.001$) and the volume of small bowel treated ($p = 0.001$). The plasma citrulline concentration correlated with clinical toxicity during the last 3 weeks of treatment. As a whole, citrulline concentration correlated better with radiation dose and volume parameters than clinical toxicity grading.

Conclusions: In patients treated with fractionated radiation therapy for abdominal or pelvic cancer sites, plasma citrulline concentration may be a simple objective marker for monitoring epithelial cell loss, a major event in acute radiation-induced small-bowel toxicity. © 2004 Elsevier Inc.

Citrulline, Assay, Small intestine, Radiotherapy.

INTRODUCTION

When treating abdominal or pelvic cancers with radiotherapy, either alone or combined with chemotherapy, the small bowel (SB) is the organ most frequently encountered as dose-limiting normal tissue, with regard to both acute and late treatment-related morbidity. A wide diversity of functional disorders have been observed after ionizing radiation, such as motility dysfunction (1, 2), changes in transepithelial transport processes (3, 4), or the absorption of various nutrients, such as carbohydrates, amino acids, proteins, vitamins, and bile acid (5–10). Acute radiation-induced small-bowel toxicity may necessitate treatment interruption, an established detrimental factor for treatment outcome (11). After radiation doses of 45–50 Gy, typically delivered during 5 weeks in 25 fractions, actuarial 5-year rates of severe stromal small-bowel injury, such as fistulas and strictures, are usually less than 5% (12). In contrast, persisting functional changes causing chronic diarrhea, malabsorption, and other symptoms associated with epithelial small-bowel damage have been reported in 40–50% of patients (5, 12–15). Both radiation dose and irradiated small-bowel volume (SBV) have been demonstrated as determinants for radiation-induced small-bowel toxicity (12, 16, 17).

Several functional changes have been correlated with an impaired absorptive capacity due to radiation-induced epithelial cell loss (9, 18–20). At present, no simple biologic marker is available that is sensitive and specific for radiation-induced small-bowel mucosal atrophy. Plasma citrulline has been suggested as a marker for functional small-

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Table 1. Treatment features

Patient	Malignancy	Radiotherapy indication	Anatomic site (most upper level field border)	Treatment schedule*
9	Bladder[†]	Vaginal and pelvic sidewall recurrence	Pelvis/PAO (L2–L3)	1.8–61.2
2	Cervix	FIGO IB	Pelvis (L5–S1)	2–46/BCT
4	Cervix	FIGO IIB, bulky	Pelvis/PAO (L2–L3)	2–46/BCT
6	Cervix[†‡]	FIGO IB2. pNO	Pelvis (L5–S1)	2–46/BCT
10	Cervix[†‡]	FIGO IB2. pN+	Pelvis (L5–S1)	2–48/BCT
13	Cervix[†‡]	FIGO IVA, bulky, pN0	Pelvis (L5–S1)	2–50/BCT
7	Kidney	Retroperitoneal recurrence	Recurrence + SM	3–39
3	Uterus	Vaginal vault recurrence	Pelvis (L5–S1)	2–70
12	Uterus	Sarcoma FIGO Stage IC. Adjuvant	Pelvis (L5–S1)	1.8–45
16	Uterus	Carcinoma FIGO Stage IC Adjuvant	Pelvis (L5–S1)	2–46
11	Ovary	Chemotherapy resistant recurrence	WAR	0.8–30.4
17	Ovary	Chemotherapy resistant recurrence	WAR	0.8–30.4
8	Prostate	pT3. Postoperative	Prostate[§] + SM	2–60
14	Prostate	pT3. Postoperative	Prostate + SM	2–60
19	Prostate	pT3. Postoperative	Prostate + SM	2–68
20	Prostate	Pelvic sidewall recurrence	Recurrence + SM	2–60
5	Rectum	Pelvic recurrence. Preoperative	Pelvis (L5–S1)	1.8–45
21	Rectum	Pelvic recurrence. Preoperative	Pelvis (L5–S1)	1.8–45
1	Testis	Seminoma Stage I. Adjuvant	PAO (T10–T11)	1.8–25.2
15	Testis	Seminoma Stage I. Adjuvant	PAO (T10–T11)	1.8–25.2
18	Testis	Seminoma Stage I. Adjuvant	PAO (T10–T11)	1.8–25.2
22	Testis	Seminoma Stage IIA. Adjuvant	PAO (T10–T11)	1.8–30.6
23	Vulva	Carcinoma. Groin recurrence	Pelvis (L5–S1)	2–60

Abbreviations: BCT = brachytherapy; PAO = para-aortic lymph node area; T, L, S = thoracic, lumbar, and sacral vertebra, respectively; SM = safety margin (typically 2 cm in all directions); WAR = whole abdominal radiation.

* Dose per fraction – Total tumor dose.

[†] Treatment combined with pelvic hyperthermia once weekly during external beam irradiation.

[‡] Retroperitoneal lymph node dissection.

[§] Prostate: preoperative prostate bed including seminal vesicles.

bowel enterocyte mass (21–26). Citrulline, a nitrogen end product of small-bowel enterocyte glutamine metabolism, accounts for almost 30% of metabolized glutamine nitrogen in the rat small intestine (27). The small-intestinal enterocyte contains specific mitochondrial enzymes involved in citrulline production but lacks the cytosolic enzymes necessary for its conversion to arginine (28–30). This unique enzymatic profile (25) and the fact that citrulline is not metabolized by the liver (31) account for the fact that the small bowel is the principal source of circulating citrulline. Consequently, the plasma citrulline concentration is highly dependent upon the intestinal cell mass (28).

We have validated the use of citrullinemia as an assay for radiation-induced small-bowel epithelial cell loss in mice after exposure to a single homogeneous dose of whole-body irradiation (26). The aim of the present study was to confirm our preclinical results in patients exposed to a fractionated inhomogeneous dose delivered to the small bowel during abdominal and/or pelvic irradiation. For this purpose, the postabsorptive plasma citrulline concentration has been measured as a marker for radiation-induced small-bowel mucosal atrophy immediately preceding treatment and weekly during treatment. In addition, citrullinemia has been measured at intervals of 2 weeks and 3 and 6 months after completion of radiotherapy to assess subacute radiation effects. Our hypothesis, based on our preclinical data (26), is that changes in citrullinemia are correlated with small-bowel dosimetric and volumetric parameters and with clinical symptoms attributable to acute small-bowel radiation toxicity.

METHODS AND MATERIALS

Subjects

This study, carried out between November 2001 and November 2002, included 23 successive patients receiving abdominal and/or pelvic irradiation (RT) at the Maastricht Radiation Oncology Clinic. Patients treated with concomitant chemotherapy or an impaired renal function (Glomerular Filtration Rate according to Cockroft formula <60 mL/min) assessed less than 4 weeks before the start of treatment were excluded. The median age of 9 male and 14 female patients was 63.1 years (range, 28.3–72.6 years). The primary tumor site, the indications for radiotherapy, and the anatomic treatment sites are summarized in Table 1.

Studied variables

In all patients the assessment of clinical toxicity and the acquisition of blood samples for plasma citrulline measurement were performed on the same day.

Table 2. Lower Gastrointestinal Radiation Therapy Oncology Group acute radiation morbidity scoring criteria

0	No change.
1	Increased frequency or change in quality of bowel habits not requiring medication.
2	Diarrhea requiring parasympatholytic drugs/abdominal pain requiring analgesics.
3	Diarrhea requiring parenteral support/abdominal distention (flat plate radiograph demonstrates distended bowel loops).
4	Acute or subacute obstruction, fistula, or perforation. GI bleeding requiring transfusion; abdominal pain requiring tube decompression or bowel diversion.
5	Any toxicity that causes death.

Clinical parameters

Each patient was seen by the responsible physician on a weekly basis during RT and at 2 weeks and at 3 and 6 months after completion of RT to assess, where appropriate, treatment-related morbidity and tumor response. At each visit, clinical symptoms attributable to SB radiation damage were recorded using the Radiation Therapy Oncology Group (RTOG) scoring criteria for acute radiation morbidity of the lower gastrointestinal tract (32) (Table 2). In contravention of the rules for this scoring system, we also applied the score at 6 months after RT. For statistical analysis, the RTOG scores per time point were used and the percentage of the total RT treatment time that a patient was free of symptoms (= % zero score).

Patients were classified according to additional treatment modalities, such as hyperthermia, and the mode of surgery performed (i.e., extraperitoneal surgery [I] or i.p. surgery immediately preceding RT [II], i.p. surgery more than 6 months before RT [III], or no abdominal surgery [IV]). In addition, patient characteristics such as age, gender, smoking status, length, and body mass index were recorded.

Treatment planning

A planning CT scan was performed in supine treatment position with full bladder instruction. Patients were instructed to drink 500 mL oral SB contrast solution 30 min before the scan. The entire abdomen was imaged in 22 patients. In Patient 5, the volume scanned was limited according to the length of the treatment fields. Adjacent 10-mm-thick transverse images were collected and transferred to a Focus treatment-planning station (C.M.S.). The large bowel was defined first on each transverse image. The SB was then defined as both opacified and unopacified individual bowel loops other than large bowel. The small-bowel outer contour was delineated on each transverse image, enabling calculation of the total small-bowel volume (Fig. 1). All contouring was done by one of the authors (M.G.) and double checked by two others (R.B., L.L.)

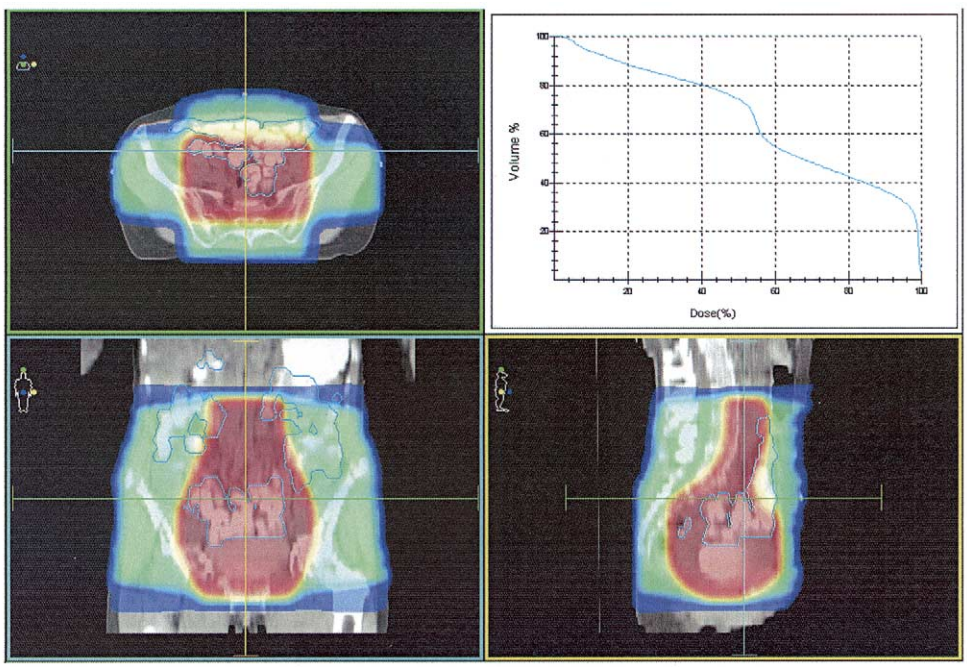


Fig. 1. Assessment of dose and volume parameters using a computed tomography scan in treatment position and 3D treatment planning software. Transverse, coronal, and sagittal plane reconstructed slices are shown, respectively. The light blue contour represents the small-bowel contour. The relative dose distribution is presented by means of a color wash with the red area representing a dose ≥50% of the prescribed tumor dose, i.e., the tissue located actually within the irradiation fields. The upper right panel shows the corresponding small-bowel dose–volume histogram. *Patient 4:* Cervix carcinoma. External radiation treatment delivered by means of 4-field box technique to the primary tumor, pelvic, and lower para-aortic lymph nodes. Upper level L2–L3, Daily fraction dose 2 Gy. Total tumor dose prescribed 46 Gy in 4½ weeks. Mean SB dose 31 Gy. SBV50% = 74%.

without knowledge of the results of citrulline measurements or dose–volume histograms (DVH). This CT-based 3-dimensional dose–volume analysis and treatment planning allowed performance of a quantitative DVH analysis for each patient. The total RT plan was used in the DVH calculation, i.e., both the dose delivered with the primary RT fields and the boost RT fields, respectively. The value of a small-bowel DVH is limited, because of variations in luminal content and small-bowel motility. However, this method seems appropriate for estimating the proportion of functional small bowel receiving a specified radiation dose (17, 33).

For all patients, the small-bowel volume receiving a dose between 5 and 55 Gy, recorded at 5-Gy intervals, and the SBV receiving at least 50% of the prescribed radiation dose (SBV50%) were calculated. For statistical analysis, volumetric parameters were expressed as the percentage of the total SBV. Thus the SBV30 is the fraction of the total amount of small bowel receiving a dose of at least 30 Gy. Dosimetric parameters used for analysis were the cumulative mean dose delivered to the entire SB during successive treatment weeks and the total mean dose delivered to the SB. Volumetric and dosimetric parameters were correlated with the RTOG toxicity score for acute SB radiation-related morbidity and with the plasma citrulline concentration at corresponding time points.

Radiation treatment

All patients were treated with megavoltage equipment (10-MV linear accelerator). Standard SB exclusion techniques, such as multiple-field techniques, where appropriate, and individual lead shielding, were used for treatment planning. The International Commission on Radiation Units and Measurement (34) reference point was chosen at the isocenter and used for dose prescription. Tumor doses prescribed to the International Commission on Radiation Units and Measurement reference point ranged between 25.2 and 70 Gy and are listed in Table 1. RT was delivered daily using 1.8–2 Gy per fraction, 5 times per week. Patient 7 was treated with 3 Gy per fraction, 4 times per week. Two patients (Patients 11 and 17) were treated with hyperfractionated whole-abdominal radiation delivering 0.8 Gy per fraction twice daily, 5 days per week. RT treatment time ranged between 3 and 6 weeks. In 5 patients treated for cervix carcinoma, RT was combined with two high-dose-rate brachytherapy applications prescribing a dose of 8.5 Gy to Manchester point A. Brachytherapy was delivered on Monday with an interval of 1 week. The first of two applications was delivered during the last week of RT or during the first week after RT, except for Patient 2, who received 2 high-dose-rate applications during the second and fourth week of RT. On days when intracavitary brachytherapy was applied, no RT was delivered. In 4 patients, RT was combined with pelvic hyperthermia once weekly (up to a total of 5 treatments).

Plasma citrulline

Postabsorptive plasma citrulline concentration was assessed on Tuesdays 15 min before irradiation. Subjects were sampled after an overnight fast and instructed to lie down for 15 min in a hospital bed, avoiding any physical exertion. On Tuesdays, irradiation was scheduled between 8:30 and 10:00 am. An antecubital vein was used for sampling 1.5 mL blood, which was collected in a heparinized cup and stored on ice. Plasma was then obtained by whole blood centrifugation at 10,000 $\times$ *g* at 4°C for 10 min. For determination of amino acids, 250 μL plasma was deproteinized by adding it to 22 mg dry 5-sulfosalicylic acid. Then it was vortexed, frozen in liquid nitrogen, and stored at −80°C until further processing. Plasma citrulline concentration (μmol/L) was measured by using high-performance liquid chromatography (35). Baseline citrulline level was assessed at Day 0. Subsequently, citrullinemia was measured weekly during RT and at intervals of 2 weeks and 3 and 6 months after RT.

Statistical analysis

SPSS for Windows software (Release 11.0) was used for statistical analysis. All results are expressed as mean $\pm$ SEM. Citrulline levels measured at different time points were compared using a paired-samples *t* test. For testing correlations between citrulline level and dose–volume and clinical parameters, Pearson correlation and one-way analysis of variance (ANOVA) were used, where appropriate. One-way ANOVA was used to test a dose and volume relationship for serum citrulline and clinical toxicity, respectively. For these tests, threshold levels for the mean SB were arbitrarily chosen at 1, 10, and 20 Gy, respectively. The choice of threshold levels for citrulline was based on a recently published correlation between citrulline level and the extent and severity of epithelial cell loss in patients with celiac and nonceliac disease (22), i.e., 10, 20, and 30 μmol/L, respectively. A p value <0.05 was considered statistically significant.

RESULTS

Clinical and dose–volume histogram parameters

Small-bowel dose–volume histogram parameters are summarized in Table 3. The mean total SBV in 22 patients was 1304 mL (range, 482–2117 mL). Body length was the only patient characteristic that was associated with the total SBV ($p = 0.056$). None of the dosimetric or volumetric parameters listed in Table 3 were associated with patient characteristics or with the type of surgery before RT (data not shown).

Citrulline concentration

All 23 patients completed RT as planned. Citrulline concentration and toxicity grading were assessed weekly, except for the first treatment week in 2 patients. At 2 weeks and 3 and 6 months after irradiation, 21, 17, and 14 patients were evaluated, respectively.

Table 3. Small-bowel dose–volume histogram parameters of all 23 patients*

DVH parameter	Mean	Range	SEM
Mean SB dose (Gy)	11.2	0–32.2	2.0
Minimum SB dose (Gy)	1.1	0–20.5	0.9
Maximum SB dose (Gy)	35.4	0–59.7	3.9
Mean SB dose 2nd RT wk (Gy)	5.0	0–17.0	0.9
Total SBV (ml)	1304	482–2117	96
SBV50% (%)	29	0–100	6.2
SBV5 (%)	37	0–100	6.5
SBV10 (%)	34	0–100	6.4
SBV15 (%)	31	0–100	6.3
SBV20 (%)	30	0–100	6.2
SBV25 (%)	24	0–94	5.7
SBV30 (%)	15	0–71	4.2
SBV35 (%)	9	0–45	3.0
SBV40 (%)	7	0–39	2.3
SBV45 (%)	4	0–31	1.6
SBV50 (%)	<1	0–5	0.3
SBV55 (%)	0	0–3	0

Abbreviations: SB = small bowel; SBV = small-bowel volume; DVH = dose–volume histogram; RT = radiotherapy SEM = standard error of the mean; SBV50% = the percentage of total SBV receiving at least 50% of the dose prescribed to the ICRU reference point; SBV5 = the percentage of total SBV receiving a dose of at least 5 Gy.

* Total SBV data available in 22 patients.

The average baseline citrulline concentration in 23 patients was 30.9 μmol/L (range, 19.1–52.9 μmol/L). During treatment, a significant decline in citrulline concentration was observed at the second ($p = 0.008$), third ($p = 0.003$), and fourth ($p = 0.014$) treatment week, respectively (Fig. 2). The maximum decline was observed during the third week of treatment (23.6 ± 2.4 μmol/L).

Seventeen of 23 patients displayed a fall in citrulline concentration during RT (mean decrease 43%, range: 14%–76%, $p = 0.005$), whereas 5 patients (Patients 1, 6, 13, 14, and 19) displayed a rise in citrulline concentration (mean increase 38%, range, 19%–64%, $p = 0.003$). The baseline citrulline concentration was lower in patients displaying an increase of citrulline concentration during treatment as compared to patients with decreasing citrulline concentration (25.2 ± 1.3 μmol/L and 32.5 ± 2.5 μmol/L, respectively), although this was not statistically significant ($p = 0.16$). In one patient, the citrulline concentration was unchanged throughout RT. None of the patient characteristics or dose–volume histogram parameters were associated with the citrulline pattern, i.e., either a fall or rise in citrulline concentration.

Citrulline: Dose–response relationship

The overall change in citrulline concentration during RT significantly correlated with the total mean SB dose (Pearson $r = -0.72$, $p < 0.001$). Citrulline concentration, categorized according to threshold values predictive for the severity and extent of villous atrophy (22), i.e. (*1*) <10 μmol/L, (*2*) 10–20 μmol/L, (*3*) 20–30 μmol/L, and (*4*) ≥30 μmol/L, respectively—were associated with different mean SB doses: 31.6 ± 0.6 Gy, 13.1 ± 2.4 Gy, 9.6 ± 2.5 Gy, and 2.7 ± 1.7 Gy for citrulline category 1, 2, 3, and 4,

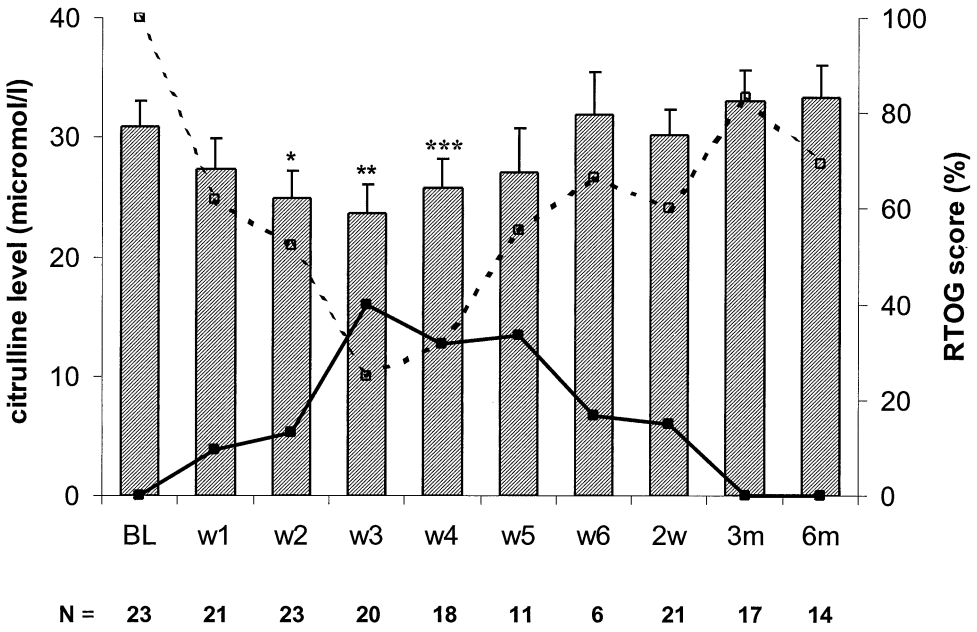


Fig. 2. Temporal pattern of plasma citrulline concentration (μmol/L) and Radiation Therapy Oncology Group (RTOG) toxicity grading relative to radiation treatment. Values for plasma citrulline level (dashed bar) represent mean ± SEM. The dashed line represents the percentage of patients with RTOG score zero; the drawn line represents the percentage of patients with RTOG score 2. Abbreviations: BL = baseline; w1–6 = radiation treatment Week 1–6; 2w, 3m, 6m = 2 weeks, 3 and 6 months postradiation. Statistics: 2-sided two-sample *t* test: **p* = 0.008, ***p* = 0.003, ****p* = 0.014.

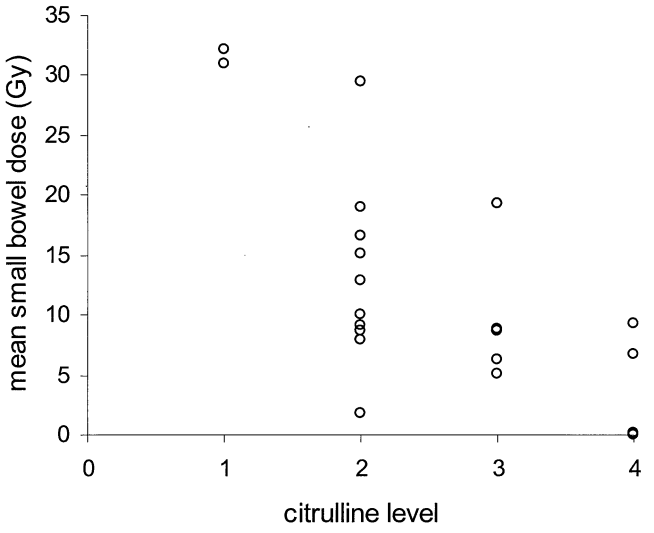


Fig. 3. Dose–effect relationship for plasma citrulline concentration categorized according to threshold values of 10, 20, and 30 μmol/L, yielding 4 groups, i.e., (*1*) <10 μmol/L, (*2*) 10–20 μmol/L, (*3*) 20–30 μmol/L, and (*4*) ≥30 μmol/L, respectively. For comparison of the mean small-bowel dose (Gy) between the respective citrulline categories, the one-way analysis of variance (ANOVA) test with Tukey's post-hoc testing is used. Data represent mean ± SEM. One-way ANOVA: $p < 0.001$. Tukey's post-hoc testing: level 1 vs. level 2, level 3, and level 4: $p = 0.005$, $p = 0.002$, and $p < 0.001$, respectively. Level 2 vs. level 3 and 4: p = nonsignificant and $p = 0.020$, respectively. Level 3 vs. level 4: p = nonsignificant.

respectively ($p < 0.001$, Fig. 3). A similar significant correlation was found during RT between the citrulline concentration and the cumulative mean SB dose: $p = 0.001$, $p < 0.001$, $p < 0.001$, and $p = 0.004$ for RT Week 2, 3, 4, and 5, respectively. No correlation was observed at the other time points during and after RT. To further explore a dose–response relationship, threshold levels for the mean SB dose of 1, 10, and 20 Gy were used and yielded similar results (Fig. 4).

Citrulline: Volume effect

The overall change in citrulline concentration during RT (μmol/L) significantly correlated with successive SBV dose levels between 5 Gy and 35 Gy at 5-Gy intervals. The volume effect was further analyzed using citrulline threshold levels of 10, 20, and 30 μmol/L, respectively. A significant volume effect was observed for the SBV dose levels between 5 Gy and 35 Gy (Fig. 5). No volume effect was found for the 2-week or 3-month and 6-month time points.

Radiation-induced small-bowel toxicity (RTOG score)

During RT, temporal changes in radiation-induced SB toxicity were observed (Fig. 2). The maximum acute SB toxicity score was 2 during the total observation time in 23 patients. Weekly assessed toxicity was correlated with the corresponding cumulative mean SB radiation dose. In addition, the fraction of total treatment time that a patient was free from symptoms (% zero score) was correlated with the total mean SB dose. For the fourth and fifth treatment week,

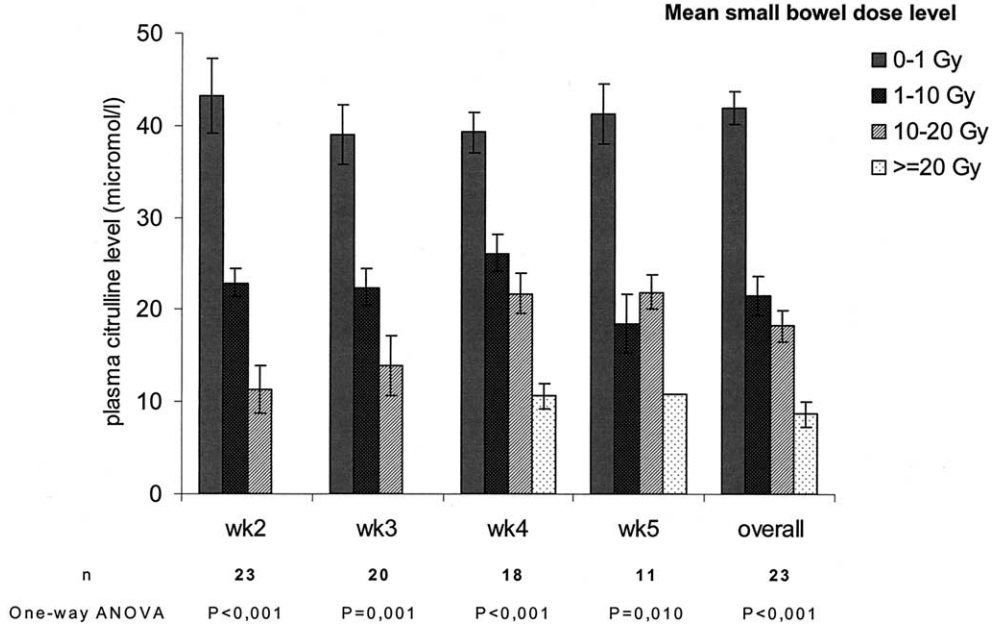


Fig. 4. Dose–effect relationship for plasma citrulline concentration at successive time points during radiotherapy. The cumulative mean dose delivered to the small bowel at each dose point is correlated with the plasma citrulline concentration (μmol/L) at the corresponding time point. Mean small-bowel doses are categorized according to 4 threshold levels, i.e., 0–1 Gy, ≥1–10 Gy, ≥10–20 Gy, and ≥20 Gy, respectively. For comparison of the mean plasma citrulline concentration between the respective threshold dose categories, the one-way analysis of variance test is used. Data represent mean ± SEM. Abbreviation: wk 2–5 = radiation week 2–5. overall = the maximum change in plasma citrulline concentration during radiotherapy treatment.

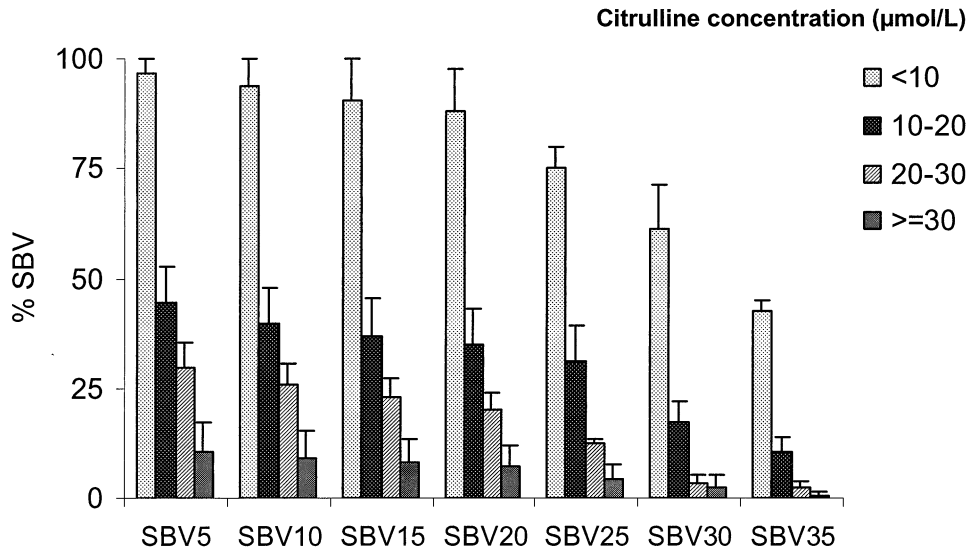


Fig. 5. Volume–effect for serum citrulline concentration. A volume effect is analyzed for small bowel volume (SBV) dose levels at 5-Gy intervals. Citrulline is categorized into 4 groups using 3 threshold levels, i.e., (*1*) <10 µmol/L, (*2*) 10–20 µmol/L, (*3*) 20–30 µmol/L, and (*4*) ≥30 µmol/L, respectively. Data represent means ± standard error of the mean (SEM). Abbreviation: SBV5 = the percentage of total small-bowel volume receiving at least 5 Gy. For statistical analysis, the one-way analysis of variance (ANOVA) test was used with Tukey's post-hoc testing. *p* values: <0.001, <0.001, <0.001, <0.001, <0.001, <0.001, and 0.045 for SBV 5–35, respectively. For SBV 5–15, the difference between each successive group is significant. For SBV20, the difference between category 2 and 3 is not significant. For SBV25–35, the difference in SBV is significant only between category 1 and 2.

the cumulative mean radiation dose significantly correlated with clinical toxicity (Pearson $r = 0.50$ and 0.69; $p = 0.033$ and $p = 0.040$, respectively). No correlation between toxicity and RT dose was found at any other time point during or after RT. The % zero score significantly correlated with the total mean SB dose ($p = 0.017$). No dose–effect relationship was observed using the maximum scored toxicity as parameter (i.e., RTOG score 2).

Weekly assessed toxicity correlated significantly with successive SBV dose levels of 5–30 Gy and 5–35 Gy during the fourth and fifth treatment week, respectively. No volume effect was found at earlier time points during RT or at later time points after treatment, respectively. The % zero score also correlated significantly with successive SBV dose levels between 5 and 30 Gy.

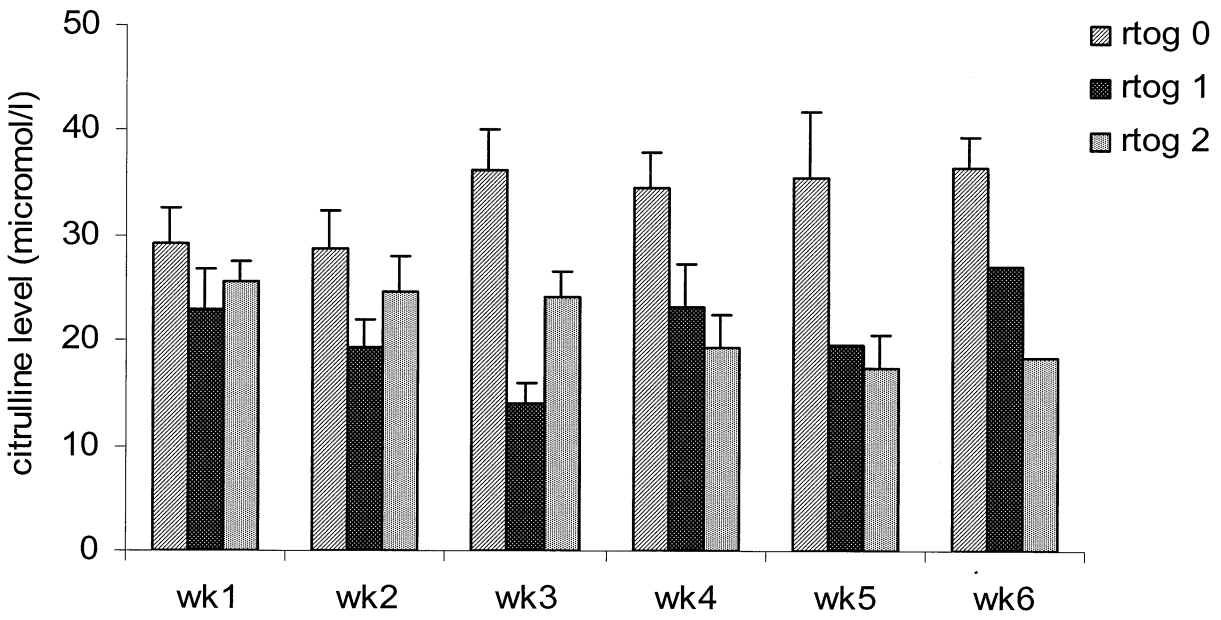
Correlation of clinical symptoms with citrulline concentration

Citrulline concentration was significantly correlated with clinical toxicity during the fourth and sixth treatment week (Pearson $r = -0.62$ and -0.86; $p = 0.007$ and $p = 0.027$, respectively), whereas a borderline significant correlation was found at the fifth treatment week ($p = 0.060$) (Fig. 6). No such relationship was observed during the first 3 weeks or after treatment. The weekly assessed % zero score significantly correlated with the citrulline concentration during treatment (Spearman's rho 0.93). Similarly, the total % zero score during treatment significantly correlated with the overall change in plasma citrulline concentration ($p = 0.002$). The % zero score correlated with the four groups of citrulline levels categorized according to threshold values of 10, 20, and 30 µmol/L, i.e., 38 ± 22%, 41 ± 7%, 53 ± 13%, and 82 ± 12% for patients with citrulline concentrations <10 µmol/L, 10–20 µmol/L, 20–30 µmol/L, and ≥30 µmol/L, respectively ($p = 0.036$). No such relationship was observed for the maximum scored toxicity (i.e., RTOG score 2) and citrulline concentration.

The limited number of patients in this study does not allow a detailed analysis on a threshold level for clinical symptoms. For that reason, we used a threshold SBV15 of 150 mL, as used for acute SB toxicity in the recently published article by Baglan *et al.* (17), although this article refers specifically to the situation of combined chemotherapy and radiotherapy in patients with rectal cancer. The % zero score during RT was 83 ± 27% as compared to 44 ± 24% for patients with an SBV15 <150 mL and ≥150 mL, respectively ($p = 0.003$). Corresponding serum citrulline concentrations were 34.2 ± 13.0 µmol/L and 18.4 ± 6.9 µmol/L, respectively ($p = 0.001$).

DISCUSSION

The present study demonstrates that plasma citrulline is a feasible marker in clinical practice to monitor radiation-induced small-intestinal mucosal atrophy in patients treated for abdominal and/or pelvic cancers and confirms preclinical results in mice after whole-body single-dose irradiation (26). Like surgery (21), celiac and nonceliac disease (22), and acute cellular rejection after small-bowel transplantation (23, 24), ionizing irradiation is an additional event



	wk1	wk2	wk3	wk4	wk5	wk6
n	21	23	20	18	9	6
One-way ANOVA	n.s.	n.s.	<0.001	0.020	n.s.	n.s.
Pearson	n.s.	n.s.	n.s.	0.007	0.060	0.027

Fig. 6. Correlation between citrulline concentration and toxicity score during radiotherapy treatment. Data for citrulline concentration (μmol/L) represent means ± standard error of the mean (SEM). Abbreviations: wk 2–6 = radiation Week 2–6, n.s. = nonsignificant; rtog = Radiation Therapy Oncology Group.

associated with reduced small-bowel epithelial cell mass that can be monitored by plasma citrulline.

The relationship between plasma citrulline concentration and epithelial cell mass has been demonstrated previously in rodents after surgical resection of small bowel (25, 36). In an experimental study using mice, we suggested plasma citrulline concentration as marker for small-bowel epithelial cell mass after a single homogeneous total-body irradiation (26). Recently, several investigators have suggested plasma citrulline as a specific marker for small-bowel functional enterocytes in patients (21–24). This is further substantiated by the present study by adding ionizing irradiation as a mechanism for (temporal) loss of functional enterocytes.

The pathophysiology of clinical symptoms related to small-bowel irradiation is complex with mucosal denudation being one of several events (37–39). In rodents, the kinetics of radiation-induced small-intestinal epithelial changes have been well described (40). Radiation damage to the intestinal crypt cell compartment and consequential epithelial denudation are strictly dose dependent (40). In patients, radiation dose, in addition to the volume of irradiated small bowel, has been demonstrated as a determinant for radiation-induced small-bowel toxicity (12, 16, 17). Plasma citrulline concentration decreased as a function of small-bowel radiation dose (Figs. 3 and 4) and volume (Fig. 5). Crenn *et al.* (22) have recently correlated plasma citrulline concentration with histologically graded villous atrophy in 42 patients with celiac and 10 patients with nonceliac villous atrophy disease. These authors identified a threshold value of 10 μmol/L (25% of the mean normal baseline value) to be predictive for severe and extensive villous atrophy and 20 μmol/L to be predictive for severe villous atrophy, whatever the extent. Based on these data, we used the same threshold levels for plasma citrulline to analyze radiation-induced effects (Figs. 3 and 5). The citrulline categories corresponded with different mean small-bowel doses (Fig. 3) and volumes treated (Fig. 5). Up to a mean small-bowel dose of 15 Gy, these threshold levels were significantly associated with the volume of small bowel treated. For higher dose levels, the 10 μmol/L threshold remains a discriminating factor for the volume of small bowel treated.

Radiation-induced small-bowel toxicity is associated with the radiation dose and the volume of small bowel treated (12, 16, 17). We found a significant dose and volume effect for radiation-induced toxicity during the fourth and fifth treatment week. In contrast to other authors (12, 16), we did not find such a relationship for late sequelae, i.e., at 6 months. However, the limited number of patients and

follow-up, respectively, do not permit conclusions on this issue.

Plasma citrulline and the RTOG toxicity score were used as end points for radiation-induced small-bowel toxicity in this study. A radiation dose and volume effect was observed for both end points. However, whereas the dose–response relationship for plasma citrulline was significant at Weeks 2–5 (Fig. 4), at Weeks 4 and 5 it was significant only for the toxicity score. Overall, citrullinemia correlates poorly with the severity of clinical symptoms as graded with the RTOG acute toxicity score used in this study, although a correlation was observed with the time that a patient is without symptoms (i.e., the % zero score). This may be explained in part by the fact that radiation-induced epithelial cell loss, which is typically measured by the plasma citrulline level, is merely one of several pathophysiological mechanisms underlying clinical symptoms (37–39). Also the fact that the clinical toxicity score, in contrast to plasma citrulline concentration, is a subjective score may further contribute to this poor correlation. The poor correlation between subjective symptoms, i.e., complaints, and objective end points for small-bowel radiation injury has been recognized before (5). Similar drawbacks exist for the use of sophisticated planning systems to calculate small-bowel dose and volume parameters. As reported by others (17, 33), we used a planning CT scan for computing a small-bowel DVH. The SBV is reconstructed from the SB contours as delineated on each transverse slice, a parameter that will depend highly on the protocol used for the CT scan, e.g., whether patients are fasted before the scan and if so, for how long? What is the volume of contrast solution administered, and what is the time interval between the contrast administration and the CT scan? A substantial variation exists in the protocols used by different authors with regard to these aspects (12, 16, 17, 41). Furthermore, without a previous history of abdominal surgery, the small bowel is a mobile organ. As a consequence, the SBV treated, as well as the dose delivered, to a particular part of SB will vary daily, not least because of a possible variation in bladder filling.

Despite several attempts to standardize registration and consequently the publication of treatment-related toxicity, most authors still use a toxicity scoring system adjusted to their own (historical) clinical practice and/or clinical question (42–44), as was also done to some extent in this study. Because of this and the fact that toxicity grading systems are being adjusted on a regular basis, an objective marker for normal-tissue toxicity, such as the plasma creatinine level is for the kidney function, would definitely be of great benefit. Ideally, such a marker should then be easily accessible and independent of medication and metabolic events such as diet and nutritional status. We did not find a correlation between baseline citrulline concentration and body mass index, surgical status, or primary malignancy. Also Crenn *et al.* (22) found normal citrulline concentrations in 10 severely malnourished patients with anorexia nervosa.

In contrast to currently used physical parameters (i.e., radiation dose and volume parameters), biologic markers (such as the citrulline concentration) provide information about individual biologic variability among patients in terms of intrinsic radiosensitivity. In the case of acute-reacting tissues such as the small-intestinal epithelium, such information might prove to be useful as a predictive assay and consequently lead to adjustments in the treatment volume and/or total radiation dose.

A rather unexpected finding was the increase of plasma citrulline concentration during RT in a substantial number of patients. The analytical method used by us is highly reproducible (coefficient of variation 3%), excluding a large variation in the measured results (35, 45). No dosimetric or volumetric parameter explained this finding; nor did any of the patient characteristics such as age or gender (46). A single plasma citrullinemia measurement immediately before the start of treatment was considered as representative baseline value in the present study. To the authors' knowledge, no data exist in the literature about the physiologic variation of plasma citrulline concentration in time. However, the mean ± SEM baseline value in the present study (30.9 ± 2.1 μM) is in complete agreement with previous findings at our laboratory in healthy volunteers under similar conditions of starvation (47). Interestingly, the mode of surgical treatment was different in patients who displayed an increased plasma citrulline concentration. Thus a rise in plasma citrulline was observed during RT in 5 of 6 patients (83%) who underwent extraperitoneal surgery before RT as compared to 0 of 17 patients with different modes of surgical intervention. An explanation for this can be only speculative. Although all patients had normal renal function before the start of RT, a temporary decline of renal function cannot be ruled out. A reactive proliferation of SB epithelium might be another possibility that would be in agreement with data so far published on citrulline as a marker for functional enterocytes (21–26, 36) and the observation that plasma citrulline concentration may change rapidly in response to altered pathophysiological conditions (24).

In summary, small bowel is an important dose-limiting normal tissue during treatment of abdominal or pelvic cancers with radiotherapy. Furthermore, combined radiotherapy with chemotherapy is being used more frequently for an increasing number of malignancies originating at abdominal or pelvic sites (48–50). Because many of the drugs used affect also the proliferative activity at the crypts, treatment-related bowel toxicity will be enhanced (48). Epithelial cell loss has been associated with radiation-induced changes in small-bowel function (9, 18–20). Mainly for practical reasons, currently available tests for SB radiation damage are not suitable for monitoring purposes during and after fractionated radiation (38). We chose citrullinemia for its methodological simplicity (21–26, 36) and the lack of methodological drawbacks, as mentioned before. As a whole, plasma citrulline seems to be a quantitative parameter independent of the underlying cause for epithelial cell loss (21–23). Consequently, this marker provides an objective parameter to the clinician,

enabling standardized assessment of treatment-related morbidity. The present study provides the proof of principle for the use of plasma citrulline—in addition to surgery (21), celiac and nonceliac disease (22), and post-transplantation cellular rejection (23, 24)—as an assay for radiation-induced small-intestinal mucosal atrophy.

REFERENCES

1. Picard C, Wysocki J, Fioramonti J, Griffiths NM. Intestinal and colonic motor alterations associated with irradiation-induced diarrhoea in rats. *Neurogastroenterol Motil* 2001;13: 19–26.
2. Fraser R, Frisby C, Blackshaw LA, Schirmer M, Howarth G, Yeoh E. Small intestinal dysmotility following abdominal irradiation in the rat small intestine. *Neurogastroenterol Motil* 1998;10:413–419.
3. Gunter Smith PJ. Gamma radiation affects active electrolyte transport by rabbit ileum: Basal Na and Cl transport. *Am J Physiol* 1986;250:G540–545.
4. Lebrun F, Benderitter M, Berroud A, Voisin P, Griffiths NM. Potential role of the membrane in the development of intestinal cellular damage after whole-body gamma irradiation of the rat. *Can J Physiol Pharmacol* 2002;80:686–693.
5. Yeoh E, Horowitz M, Russo A, *et al.* A retrospective study of the effects of pelvic irradiation for carcinoma of the cervix on gastrointestinal function. *Int J Radiat Oncol Biol Phys* 1993; 26:229–237.
6. Travis S, Menzies I. Intestinal permeability: Functional assessment and significance. *Clin Sci* 1992;82:471–488.
7. Craig RM, Atkinson AJ, Jr. D-xylose testing: A review. *Gastroenterology* 1988;95:223–231.
8. Thomson AB, Cheeseman CI, Walker K. Effect of abdominal irradiation on the kinetic parameters of intestinal uptake of glucose, galactose, leucine, and gly-leucine in the rat. *J Lab Clin Med* 1983;102:813–827.
9. Juby LD, Dixon MF, Axon AT. Abnormal intestinal permeability and jejunal morphometry. *J Clin Pathol* 1987;40:714–718.
10. Thomson AB, Cheeseman CI, Walker K. Intestinal uptake of bile acids: Effect of external abdominal irradiation. *Int J Radiat Oncol Biol Phys* 1984;10:671–685.
11. Petereit DG, Sarkaria JN, Chappell R, *et al.* The adverse effect of treatment prolongation in cervical carcinoma. *Int J Radiat Oncol Biol Phys* 1995;32:1301–1307.
12. Letschert JG, Lebesque JV, Aleman BM, *et al.* The volume effect in radiation-related late small bowel complications: Results of a clinical study of the EORTC Radiotherapy Cooperative Group in patients treated for rectal carcinoma. *Radiother Oncol* 1994;32:116–123.
13. Yeoh E, Razali M, O'Brien PC. Radiation therapy for early stage seminoma of the testis. Analysis of survival and gastrointestinal toxicity in patients treated with modern megavoltage techniques over 10 years. *Australas Radiol* 1993;37:367–369.
14. Yeoh E, Horowitz M, Russo A, *et al.* Effect of pelvic irradiation on gastrointestinal function: A prospective longitudinal study. *Am J Med* 1993;95:397–406.
15. Yeoh E, Horowitz M, Russo A, Muecke T, Robb T, Chatterton B. The effects of abdominal irradiation for seminoma of the testis on gastrointestinal function. *J Gastroenterol Hepatol* 1995;10:125–130.
16. Gallagher MJ, Brereton HD, Rostock RA, *et al.* A prospective study of treatment techniques to minimize the volume of pelvic small bowel with reduction of acute and late effects associated with pelvic irradiation. *Int J Radiat Oncol Biol Phys* 1986;12:1565–1573.
17. Baglan KL, Frazier RC, Yan D, Huang RR, Martinez AA, Robertson JM. The dose-volume relationship of acute small bowel toxicity from concurrent 5-FU-based chemotherapy and radiation therapy for rectal cancer. *Int J Radiat Oncol Biol Phys* 2002;52:176–183.
18. Overgaard J, Matsui M. Effect of radiation on glucose absorption in the mouse jejunum in vivo. *Radiother Oncol* 1990;18: 71–77.
19. Kirichenko AV, Mason KA, Straume M, Teates CD, Rich TA. Nuclear scintigraphic assessment of radiation-induced intestinal dysfunction. *Radiat Res* 2000;153:164–172.
20. Gunter Smith PJ. Gamma radiation affects active electrolyte transport by rabbit ileum. II. Correlation of alanine and theophylline response with morphology. *Radiat Res* 1989;117: 419–432.
21. Crenn P, Coudray Lucas C, Thuillier F, Cynober L, Messing B. Postabsorptive plasma citrulline concentration is a marker of absorptive enterocyte mass and intestinal failure in humans. *Gastroenterology* 2000;119:1496–1505.
22. Crenn P, Vahedi K, Lavergne Slove A, Cynober L, Matuchansky C, Messing B. Plasma citrulline: A marker of enterocyte mass in villous atrophy-associated small bowel disease. *Gastroenterology* 2003;124:1210–1219.
23. Gondolesi G, Fishbein T, Chehade M, *et al.* Serum citrulline is a potential marker for rejection of intestinal allografts. *Transplant Proc* 2002;34:918–920.
24. Pappas PA, Saudubray JM, Tzakis AG, *et al.* Serum citrulline as a marker of acute cellular rejection for intestinal transplantation. *Transplant Proc* 2002;34:915–917.
25. Wakabayashi Y, Yamada E, Yoshida T, Takahashi N. Effect of intestinal resection and arginine-free diet on rat physiology. *Am J Physiol* 1995;269:G313–318.
26. Lutgens LC, Deutz NEP, Gueulette J, *et al.* Citrulline: A physiological marker enabling quantitation and monitoring of epithelial radiation induced small bowel damage. *Int J Radiat Oncol Biol Phys* 2003;57:1067–1074.
27. Windmueller HG. Glutamine utilization by the small intestine. *Adv Enzymol Relat Areas Mol Biol* 1982;53:201–237.
28. Wakabayashi Y, Yamada E, Hasegawa T, Yamada R. Enzymological evidence for the indispensability of small intestine in the synthesis of arginine from glutamate. I. Pyrroline-5-carboxylate synthase. *Arch Biochem Biophys* 1991;291:1–8.
29. Wakabayashi Y, Yamada E, Yoshida T, Takahashi H. Arginine becomes an essential amino acid after massive resection of rat small intestine. *J Biol Chem* 1994;269:32667–32671.
30. Wu G. Synthesis of citrulline and arginine from proline in enterocytes of postnatal pigs. *Am J Physiol* 1997;272:G1382–1390.
31. Windmueller HG, Spaeth AE. Source and fate of circulating citrulline. *Am J Physiol* 1981;241:E473–480.
32. Cox JD, Stetz J, Pajak TF. Toxicity criteria of the Radiation Therapy Oncology Group (RTOG) and the European Organization for Research and Treatment of Cancer (EORTC). *Int J Radiat Oncol Biol Phys* 1995;31:1341–1346.
33. Das IJ, Lanciano RM, Movsas B, Kagawa K, Barnes SJ. Efficacy of a belly board device with CT-simulation in reducing small bowel volume within pelvic irradiation fields. *Int J Radiat Oncol Biol Phys* 1997;39:67–76.
34. International Commission on Radiation Units and Measurements (ICRU). Report Number 50. Prescribing, recording and reporting photon beam therapy. Washington, DC: ICRU; 1993.
35. van Eijk HM, Rooyakkers DR, Deutz NE. Rapid routine determination of amino acids in plasma by high-performance

liquid chromatography with a 2–3 microns Spherisorb ODS II column. *J Chromatogr* 1993;620:143–148.
36. Chen K, Nezu R, Sando K, *et al.* Influence of glutamine-supplemented parenteral nutrition on intestinal amino acid metabolism in rats after small bowel resection. *Surg Today* 1996;26:618–623.
37. Thomson AB, Keelan M, Thiesen A, Clandinin MT, Ropeleski M, Wild GE. Small bowel review: Diseases of the small intestine. *Dig Dis Sci* 2001;46:2555–2566.
38. Griffiths NM. The example of gastrointestinal damage induced by ionising radiation: Are there accessible markers? *Cell Mol Biol* 2001;47:427–435.
39. MacNaughton WK. Review article: New insights into the pathogenesis of radiation-induced intestinal dysfunction. *Aliment Pharmacol Ther* 2000;14:523–528.
40. Potten CS. Structure, function and proliferative organisation of mammalian gut. Amsterdam: Elsevier Science B.V.; 1995; p. 1–31.
41. Herbert SH, Solin LJ, Hoffman JP, *et al.* Volumetric analysis of small bowel displacement from radiation portals with the use of a pelvic tissue expander. *Int J Radiat Oncol Biol Phys* 1993;25:885–893.
42. Miller AR, Martenson JA, Nelson H, *et al.* The incidence and clinical consequences of treatment-related bowel injury. *Int J Radiat Oncol Biol Phys* 1999;43:817–825.
43. Miller RC, Sargent DJ, Martenson JA, *et al.* Acute diarrhea during adjuvant therapy for rectal cancer: A detailed analysis from a randomized intergroup trial. *Int J Radiat Oncol Biol Phys* 2002;54:409–413.
44. Mundt AJ, Lujan AE, Rotmensch J, *et al.* Intensity-modulated whole pelvic radiotherapy in women with gynecologic malignancies. *Int J Radiat Oncol Biol Phys* 2002;52:1330–1337.
45. van Eijk HM, Huinck MP, Rooyakkers DR, Deutz NE. Automated simultaneous isolation and quantitation of labeled amino acid fractions from plasma and tissue by ion-exchange chromatography. *J Chromatogr B Biomed Appl* 1994;660: 251–257.
46. van Eijk HM, de Jong CHM, Deutz NEP, Soeters PB. Influence of storage conditions on normal plasma amino-acid concentrations. *Clin Nutr* 1994;13:374–380.
47. van Eijk HM, van der Heijden MA, van Berlo CL, Soeters PB. Fully automated liquid-chromatographic determination of amino acids. *Clin Chem* 1988;34:2510–2513.
48. Green JA, Kirwan JM, Tierney JF, *et al.* Survival and recurrence after concomitant chemotherapy and radiotherapy for cancer of the uterine cervix: A systematic review and meta-analysis. *Lancet* 2001;358:781–786.
49. Han SC, Kim DH, Higgins SA, Carcangiu ML, Kacinski BM. Chemoradiation as primary or adjuvant treatment for locally advanced carcinoma of the vulva. *Int J Radiat Oncol Biol Phys* 2000;47:1235–1244.
50. Pahlman L, Hohenberger W, Gunther K, Fietkau R, Metzger U. Is radiochemotherapy necessary in the treatment of rectal cancer? *Eur J Cancer* 1998;34:438–448.